Indian J. Plant Physiol., Vol. 10, No. 1, (N.S.) pp. 14-19 (Jan.-Mar., 2005)

IN VITRO SELECTION AND CHARACTERIZATION OF WATER STRESS TOLERANT CULTURES OF BELL PEPPER

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Received on 19 July, 2004, Revised 15 Dec., 2004

SUMMARY

Callus of bell pepper (*Capsicum annuum* L.) was initiated from hypocoty1 on MS medium supplemented with NAA (0.5mg\1) and BAP (0.2mg\1). For proliferation of callus the hormone concentrations were reduced to half. Cell clumps of about 1mm diameter were exposed to increasing concentration of polyethylene glycol (PEG) ranging from 10 g\1 to 100 g\1 for water stress tolerance. Upon incubation for 30 days, the cells, which could tolerate this concentration of PEG, grew to form calli. Selected calli were further subcultured on to the selective medium (100 g\1) PEG for 8 weeks and then transferred to normal MS medium for proliferation. The selected calli when transferred from the normal to the selective medium , were capable of growing on it. Although, there were difference in their growth, the pattern was sigmoidal in both the cell lines. Compared to the control, selected cells contained significantly higher levels of soluble proteins, total sugars, reducing sugar, and free amino acids. The water stress tolerant cells also revealed enhanced activities of enzymes, malate dehydrogenase, alkaline invertase, NADP+ - isocitrate dehydrogenase, aspartate amino transferase, glutamate pyruvate transaminase and acid phosphatase.

***Key wards*: callus culture, enzymes, water stress.**

INTRODUCTION

Water stress is the major problem in agriculture and the ability to withstand such stress is of immense economic importance. Water stress tolerance involves subtle changes in cellular biochemistry. It appears to be the result of accumulation of compatible solutes and of specific proteins that can be rapidly induced by osmotic stress (Rhodes 1987). Many workers like Sabbah and Tal (1990), Srivastava *et al*. (1995) reported different approaches to select cells with relaxed feedback mechanism under *in vitro* conditions. A potential application of such studies is the subsequent regeneration of tolerant plants (Siddeswar and Kavikishore 1989).

The present study deals with *in vitro* selection of water stress tolerant callus cultures of bell pepper using PEG in the medium. The selected cells have been characterised for growth behaviour, level of free amino acids, free proline, soluble protein, total sugar, reducing sugars, activities of malate dehydrogenase, alkaline invertase, NADP+ isocitrate dehydrogenase, aspartate amino transferase, glutamate pyruvate transaminase and acid phosphatase.

MATERIALS AND METHODS

Callus cultures of bell pepper (*capsicum annuum* L.) cv. Californiа wonder were initiated from hypocotyl segments of aseptically grown seedlings. Explants of 0.5-

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1.0 cm were cultured on the MS medium (Murashige & Skoog 1962) supplemented with NAA (0.5 mg\1) and BAP (0.2 mg\1). All the cultures were incubated under 5000 lux at 25±2ºC with 16 h photoperiod. For subculturing, concentrations of the hormone were reduced to half. The calli were subcultured at an interval of 4 weeks.

Cell clumps of about 1 mm in diameter were exposed to different concentration of polyethylene glycol (PEG-6000) ranging from 10 g\1 to 120 g\1. The cells were selected at a concentration of 100 g\1 PEG. They were subcultured on the same selective medium for 6 weeks to check their stability and then transferred on to normal MS medium (0.25 mg\1 NAA and 0.1 mg\1 BAP) without PEG for further proliferation. Selected and non- selected callus were characterized for their growth behaviour, contents of soluble proteins, proline, total free amino acids, total sugars, reducing sugars and activities of malate dehydrogenase, alkaline invertase, NADP+ -isocitrate dehydrogenase, aspartate amino transferase, glutamate pyruvate transminase and acid phosphatase.

The growth of *in vitro* selected and non-selected cell lines (control) were studied on MS medium supplemented with NAA (0.25 mg\1) and BAP (0.1 mg\1) and selective MS medium containing 100 g\1 PEG in addition to NAA (0.25 mg\1) and BAP (0.1 mg\1). The callus was cut into small pieces. Three pieces of callus each weighing approximately 159± 0.2 mg were cultured in each flask. Subsequently at 10 days interval the callus from the flasks was removed and its fresh weight was determined. It was then oven dried at 80ºC for 48 hours till constant dry weight. This procedure was repeated till 40 days after inoculation.

For biochemical studies samples used were of selected and non-selected callus growing on normal medium. Free proline was estimated as described by Bates *et al*. (1973). Free amino acids and soluble sugars were extracted from 1 g of dried selected and non-selected callus with hot 80% ethanol on a boiling water bath. The extraction was repeated thrice and the supernatants were pooled. After evaporation to dryness on a water bath, the residue was dissolved in 20% ethanol to a known volume and centrifuged. The clear supernatant was used for estimations. Total sugars were estimated using phenol sulphuric method (Dubois *et al*. 1956). Reducing sugars was estimated as described by Nelson (1944). Total free amino acids were determined using ninhydrin (Lee and Takhahashi 1966).

Enzymes were extracted by homogenising the fresh tissue in a chilled pestle and mortar with phosphate buffer (100 mM, pH 7.5) containing 10 mM KCl, 1 mM $MgCl_2$, 10mM EDTA and 10 mM 2-mercaptoethanol. The extract for acid phosphatase was prepared in Tris HCl buffer (100 mM, pH 7.5) containing 10 mM 2-mercaptoethanol. The homogenate was centrifuged at 10,000 g for 20 minutes and the resulting supernatant was used for the estimation of total soluble protein and for the assay of enzyme activities.

The proteins in the above extracts were precipitated with equal volume of 20% TCA. The samples were placed at 4ºC for 30 minutes and centrifuged at 5000g for 15 minutes. The precipitates were washed with acetone thrice and dissolved in 0.1N NaOH. Estimations of soluble proteins were done as described by Lowry *et al*. (1951). Acid phosphatase was assayed using p-nitrophenylphosphate as substrate (Jones 1969). The enzyme activity was expressed as EU g^{-1} callus dry weight . One enzyme unit was defined as the change in absorbance at 540 nm in 0.01 min.

Alkaline invertase was assayed as described by Morell and Copeland (1984). The enzyme activity was expressed µmol of reducing sugars formed min^{-1} g^{-1} dry weight.

Measuring NADH oxidized at 340 nm assayed the activity of malate dehydrogenase (Sudhakar *et al*. 1991). The activity was expressed as µmol of NADH oxidized min^{-1} g^{-1} dry weight. The activity of $NADP^+$ -isocitrate dehydrogenase was assayed by following the rate of increase in absorbance due to NADP+ reduction at 340 nm (Gupta and Singh 1988). The enzyme activity was expressed as nmol of $NADP^+$ reduced min^{-1} g^{-1} dry weight. The activity of glutamate pyruvate transminase was determined by coupling the alanine amino transferase catalysed production to lactate dehydrogenase and activity for aspartate amino transferase was determined using a NADH dependent malate dehydrogenase linked reaction (Bergmeyer and Bernt 1974). The enzyme activity was

expressed as nmol\µmol of NADH oxidisde min^{-1} g^{-1} dry weight.

RESULTS AND DISCUSSION

The cells were selected at 100 g\1 polyethylene glycol (PEG-6000). Upon incubation for 30 days, most of the callus died but only a few cells, which could tolerate this concentration of PEG, grew to form cell clones. Selected clones were subcultured on the selective medium for 8 weeks and then transferred to the normal MS medium. To check the stability of tolerance at cellular level selected calli were transferred from the normal to the selective medium (with the same concentration of PEG). The selected clones were capable of growing on it.

The non-selected callus showed a higher growth rate on normal medium than the selected callus and the growth curve showed a sigmoid pattern (Table 1). This may be attributed to the fact that the selected callus was under stress condition and so when it was transferred to the normal medium, the growth reduced as a results of time taken for acclimatization to the normal medium. However, selected callus grew better on selective medium than non-selected callus which showed a continuous decrease in fresh weight. This may be due to stress condition that enabled selected callus to adapt better.

Compared to control, the selected cells showed higher content of total soluble and reducing sugars (Table 2). Accumulation of sugars in response to PEG induced stress has been shown earlier (Srivastava *et al*. 1995, Purushotham *et al*. 1998, Narang *et al*. 1998). The sugars might contribute to water stress tolerance either by serving as osmotica or as respiratory substrates.

The selected clones contained nearly twice the soluble protein as compared to control (Table 1). Increase in soluble protein in response to PEG induced stress has

Table 1. Comparison of fresh weight (mg) of non-selected (control) and selected callus on normal MS medium and selected callus on selective medium containing 100 g/1 PEG*.

Days	Treatment		
	Normal MS medium		Selective MS medium
	Selected clones	Control	Selected clones
0	159±2.0	159±2.0	159±2.0
10	258±3.1	330±3.5	249±4.51
20	451±3.5	610±5.0	410±3.10
30	710±4.0	992±6.6	791±5.90
40	982±6.6	1680±7.6	909±2.50

* Each value is mean of six replications

Table 2. Comparison of levels of biochemical constituents in control and *in vitro* selected clones of bell pepper*.

Biochemical parameters	Treatment		t-value
	Control	Selected clones	
Proline µg g^{-1} dry wt	451±1.03	1038±5.16	107.2
Amino acids mg g^{-1} dry wt	12.26±0.26	19.63±0.26	30.70
Reducing sugars mg g^{-1} dry wt	23.58±0.48	34.77±0.51	21.27
Total sugars mg g^{-1} dry wt	64.58±0.51	93.47±0./78	36.38
Soluble protein mg g^{-1} dry wt	51.44±0.36	106±2.58	12.27

* Each value is mean of six replications
t table value at 10 degree of freedom and at 1% level of significance is 3.17 and 5% level of significance is 2.23

been shown earlier in crops like tomato, tobacco and chilli (Srivastava *et al*. 1995, Quintero- Higuera-Fernanada *et al.* 1997). Stress adapted cell lines of tomato showed a decline in protein levels (Handa *et al*.1983). It was suggested that a small portion of protein released by the stressed cells are involved in nutrient transport and other proteins are important component of the membrance structure, providing cells the ability to withstand high degree of stress (Handa *et al.* 1982).

There was significantly higher accumulation of free amino acids including proline in the selected cells. The levels of free amino acids and proline were 1.6 and 2.3 fold higher in selected cells as compared to the control (Table 2). The increase in the level of proline was higher than the free amino acids. This suggested that the mechanism of generation of free proline in the selected cells may be independent of that regulating the level of other amino acids. Enhanced level of free amino acids and proline in water stress tolerant cells have been reported earlier in other crops also (Purushotham *et al.* 1998). The increase in the level of proline could be due to *de novo* synthesis or protein hydrolysis. *De novo* synthesis could account for an increase in free proline as soluble proteins did not decrease in water stress tolerant cells. Ability to accumulate proline has been used as a basis for selection of drought tolerance in several species (Stajner *et al.* 1995, Van Heerden *et al.* 1996). Proline could be involved in protection of plant tissue against stress by acting as N-storage compound, osmosolute and hydrophobic protectant for enzymes and subcellular organelles (Handa *et al.* 1986, Lerudulier *et al*. 1984). In the present studies amino acid content increase because of the continued synthesis. Higher amino content enables the plant to sustain better growth possibly by maintaining the water of the plant or by supplying essential amino acid protein synthesizing machinery drought (Bewley 1973). Increase in the free amino acid did not occur at the expense of soluble proteins in the present studies. Enhanced level of free amino acids has been reported in response to PEG induced water stress in other crops like tomato, groundnut etc. (Srivastava *et al*. 1995, Purushotham et al. 1998).

There was a 1.22 fold increase in activity of malate dehydrogenase in water stress tolerant cell lines of bell pepper (Table 3). Sudhakar *et al*. (1991) and Srivastava *et al*. (1995) reported increase in activity during stress in horse gram seedling and tomato. In the selected lines higher level of malate dehydrogenase is necessary for the biosynthesis of osmotic solutes and for processes such as ion transport, which contributes to the survival of cells under water stress (Srivastava *et al.* 1995). Handa et al. (1983) reported increase in malate organic anion as cells became adapted to increasing level of water stress. The activity of alkaline invertase was 1.80 fold higher in water stress tolerant cell lines of bell pepper. In contrast low activity of alkaline invertase has been reported by in both leaves and roots of stressed wheat plants (Narang *et al*. 1998). There was a 2.35 fold increase in activity of $NADP^+$ isocitrate dehydrogenase in water stress tolerant cell lines of bell pepper. This may be due to the increase

Table 3. Comparison of enzyme of activities in control and *in vitro* selected clones of bell pepper*.

Enzymes	Treatment		t-value
	Control	Selected clones	
Malate dehydrogenase (μmol min^{-1} g^{-1} dry wt)	130.26±1.29	158.67±1.03	16.54
Alkaline invertase (μmol min^{-1} g^{-1} dry wt)	10.36±0.26	18.63±0.26	21.88
$NADP^+$ - isocitrate dehydrogenase (μmol min^{-1} g^{-1} dry wt)	201.70±1.55	437.60±2.58	86.98
Asparate amino transferase (μmol min^{-1} g^{-1} dry wt)	257.70±1.55	303.89±0.78	14.84
Glutamate pyruvate transaminase (nmol min^{-1} g^{-1} dry wt)	2855±25.8	5031±36.5	77.46
Acid phosphatase (EU min^{-1} g^{-1} dry wt ; EU change in absorbance at 540nm of 0.01 min^{-1})	2157.87±25.8	3789.47±2.58	60.49

* Each value is mean of six replications

t table value at 10 degree of freedom and at 1% level of significance is 3.17 and 5/5 level of significance is 2.23

in the organic anions such as citrate , as the cells become adapted to the increasing levels of the water stress (Handa *et al.* 1983). Sudhakar *et al.* (1991) reported increase in isocitrate dehydrogenase activity during stress in horse gram seedlings. Aspartate amino transferase activity was found to be 1.18 fold higher in water stress tolerant clones of bell pepper. The activity of glutamate pyruvate tranaminase was 1.76 fold higher in the water stress selected clones of bell pepper. The increase in amination and transamination visualize significance of amino acids under water stress conditions (Sharma and Garg 1985). The increase in aspartate amino transferase activity with the increasing stress has been reported in crops such as soyabean nodules, wheat etc (Sharma and Garg 1985, Boland *et al*. 1982). Kaur *et al.* (1985) reported low activity of this enzyme in mungbean nodules. The activity of acid phosphatase was 1.75 fold higher in water stress tolerant clones of bell pepper. Acid phosphatase is an important enzyme in the metabolism of phosphate compounds in plants. Acid phosphatase activity increased with increasing the stress in *Dodonia viscose*, tomato etc. (Srivastava *et al.* 1995, Thakur 1991).

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